MPG-8

SLW 4/10/03 Human K^+ ion EAG Channels.

~~Novel human K^+ ion channel and therapeutic applications thereof~~

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The present invention relates to a novel human K^+ ion channel, to nucleic acid molecules encoding the same and to vectors comprising said nucleic acid molecules. The invention additionally relates to antibodies specifically directed to the novel K^+ ion channel and to pharmaceutical compositions and diagnostic kits containing at least one of the above-mentioned components. Furthermore, the present invention relates to methods of treating a disease caused by malfunction of the polypeptide of the present invention or by the (over)expression of the nucleic acid molecule of the invention comprising administering an inhibitor of said (over)expression or of ion channel function or an inhibitor abolishing said malfunction to a patient in need thereof. Methods of devising drugs for treating or preventing the above-mentioned disease, methods of inhibiting cell proliferation and methods of prognosing cancer are additional embodiments comprised by the present invention. The invention also envisages specific antisense or gene therapies on the basis of the nucleic acid molecule of the invention for inhibiting undesired cellular proliferation, for example, in connection with cancer or in neurodegenerative diseases.

Potassium channels are a relevant factor in the regulation of the resting potential of cells, and this has been regarded as their major role in excitable and non-excitable tissues. On the other hand, the explanation for their ubiquitous presence and the impressive variability in their properties remains elusive. A reasonable hypothesis is that potassium channels are present in all cell types because they have in addition some "housekeeping" role, for example in cell proliferation[1]. Their implication in the regulation of the cell division cycle has been tested repeatedly, and some experimental evidence has been presented[2,3]. However, especially since both depolarization and hyperpolarization of the membrane potential during cell cycle have been reported as depending on cell type[1,4], there is no general model to explain the function of potassium

channels in cell cycle. Two mechanisms have been proposed to explain the role of K^+ channels: they either influence the intracellular Ca^{2+} concentration, or control cell volume (17, 18). Both mechanisms would indirectly influence cell proliferation. A member of the *eag* family has also been proposed to be preferentially expressed in cancer cells (19) Several potassium channel blockers have been tested for their capability to block cancer cell proliferation, and some of them have even been used as coadjuvants for tumor chemotherapy, specially in multidrug-resistant tumors. Nevertheless, the lack of identification of a particular potassium channel directly involved in the control of cell proliferation has, up to date, precluded the description of more specific and effective treatment protocols.

Thus, the technical problem underlying the present invention was to identify a biological component within the conglomerate of potassium channels with their various effects on cell cycle division that allows an unambiguous assignment to cellular proliferation, with a specific view to human cellular proliferation. The solution to said technical problem is achieved by providing the embodiments characterized in the claims.

Accordingly, the present invention relates to a nucleic acid molecule comprising a nucleic acid molecule encoding a (poly)peptide having a function of the human K^+ ion *eag* channel which is

(a) a nucleic acid molecule comprising a nucleic acid molecule encoding the polypeptide having the amino acid sequence of SEQ ID: No 3 or 4;

(b) a nucleic acid molecule comprising the nucleic acid molecule having the DNA sequence of SEQ ID: No 13 or 14;

(c) a nucleic acid molecule hybridizing to the complementary strand of a nucleic acid molecule of (a) or (b); or

(d) a nucleic acid molecule being degenerate to the sequence of the nucleic acid molecule of (c).

The nucleic acid molecule of the invention encodes a (poly)peptide which is or comprises the human homologues of the rat *eag* channel. In this regard the term "a nucleic acid molecule comprising a nucleic acid molecule encoding a (poly)peptide having a function of the human K^+ ion *eag* channel" may mean that said first mentioned

nucleic acid molecule solely encodes said (poly)peptide. Thus, it may be identical to said second mentioned nucleic acid molecule. Alternatively, it may comprise regulatory regions or other untranslated regions. In a further embodiment, said first mentioned nucleic acid may comprise heterologous nucleic acid which may encode heterologous proteinaceous material thus giving rise, e.g., to fusion proteins. It is further to be noted that the DNA sequences of SEQ ID NO: 13 and 14 are splice variants of the nucleic acid sequence encoding the (poly)peptide of the invention. The corresponding amino acid sequences are depicted in SEQ ID NO: 3 and 4.

The term "having a function of a human K^+ ion *eag* channel", as used in connection with the present invention, has the following meaning: The channel has a single channel conductance in asymmetrical potassium, at 0mV of about 6 pS. This value clearly distinguishes the human channel from the rat channel for which a value of about 7 pS was measured. In addition or in the alternative, the above term may have the following meaning: The channel has a IC50 of about 1mM to quinidine when expressed in Xenopus laevis oocytes, as compared to 400µM for r*eag*. Further, when measuring voltage-dependence of activation in high extracellular potassium using a two-electrode voltage-clamp it was found that in a conductance-voltage plot, the voltage for half-activation is shifted by about 40mV or more to the right in the h*eag* channel with respect to the r*eag* channel (see Figure 13). On the basis of the above features, either alone or in combination, a differentiation based on function between the human ion channel of the invention and the prior art channels, in particular of the rat ion channel, is possible for the person skilled in the art without further ado. Preferably, the channel has all recited functions. The above values refer to values that are obtainable with the experimental set-up described in this specification. Alterations of experimental parameters such as the employment of a different expression system may, as is well known to the person skilled in the art, also change the above values. Yet, these embodiments are also comprized by the scope of the present invention.

The term "hybridizing" as used in accordance with the present invention relates to stringent or non-stringent hybridization conditions. Preferably, it relates to stringent

conditions. Said hybridization conditions may be established according to conventional protocols described, for example, in Sambrook, "Molecular Cloning, A Laboratory Manual", Cold Spring Harbor Laboratory (1989) N.Y., Ausubel, "Current Protocols in Molecular Biology", Green Publishing Associates and Wiley Interscience, N.Y. (1989), or Higgins and Hames (eds) "Nucleic acid hybridization, a practical approach" IRL Press Oxford, Washington DC, (1985). Hybridizing molecules or molecules falling under alternative (d), supra, also comprise fragments of the molecules identified in (a) or (b) wherein the nucleotide sequence need not be identical to its counterpart in SEQ ID 13 or 14, said fragments having a function as indicated above.

An example of one such stringent hybridization condition is hybridization at 4XSSC at 65 °C, followed by a washing in 0.1XSSC at 65 °C for one hour. Alternatively, an exemplary stringent hybridization condition is in 50 % formamide, 4XSSC at 42 °C. Examples of such non-stringent hybridization conditions are 4XSSC at 50 °C or hybridization with 30-40 % formamide at 42 °C. Complementary strands of hybridizing molecules comprise those which encode fragments, analogues or derivatives of the polypeptide of the invention and differ, for example, by way of amino acid and/or nucleotide deletion(s), insertion(s), substitution(s), addition(s) and/or recombination(s) or any other modification(s) known in the art either alone or in combination from the above-described amino acid sequences or their underlying nucleotide sequence(s). Using the PESTFIND program (Rogers, Science 234 (1986), 364-368), PEST sequences (rich in proline, glutamic acid, serine, and threonine) can be identified, which are characteristically present in unstable proteins. Such sequences may be removed from the polypeptide of the invention in order to increase the stability and optionally the activity of the proteins. Methods for introducing such modifications in the nucleic acid molecules according to the invention are well-known to the person skilled in the art. The invention also relates to nucleic acid molecules the sequence of which differs from the nucleotide sequence of any of the above-described nucleic acid molecules due to the degeneracy of the genetic code. All such fragments, analogues and derivatives encoding the protein of the invention are included within the scope of the present invention, as long as the essential characteristic immunological and/or biological properties as defined above remain unaffected in kind, that is the novel nucleic acid molecules of the invention include all

nucleotide sequences encoding proteins or peptides which have at least a part of the primary structural conformation for one or more epitopes capable of reacting with antibodies to said polypeptide which are encoded by a nucleic acid molecule as set forth above and which have comparable or identical characteristics in terms of biological activity. Part of the invention is therefore also concerned with nucleic acid molecules encoding a polypeptide comprising at least a functional part of the above identified polypeptide encoded by a nucleic acid sequence comprised in a nucleic acid molecule according to the invention.

The present inventors have recently described a potassium channel (*reag*) which is strongly downregulated immediately after the activation of cyclin dependent kinases (key molecules in the cell cycle regulation), in both G1-S and G2-M transitions[5]. The K^+ current is inhibited following activation of cyclin-dependent kinases due to a voltage-dependent sodium block, which is not apparent in all phases of the cell cycle. The experiments presented here are aimed to determine whether *eag*, in addition to being regulated by the cell cycle, is also able to directly influence cell proliferation and growth (20). In accordance with the present invention and with a view to the development of a suitable system for assessing (disease-related) proliferation in human cells, it was further attempted to study whether the implication of the channel in the cell cycle goes in both directions, such that it is not only *regulated by* but also *regulator of* the progression of the cell cycle.

The results obtained in this rat derived ion channel system show that in three different cell lines obtained from different species (Chinese hamster -CHO-, human -HEK293-, and mouse -NIH3T3-), the rate of proliferation is faster when the channel is overexpressed after transfection of the cells with a plasmid containing the channel DNA under the control of the cytomegalovirus promoter. Figure 1 and Figure 18a show the increase in metabolic activity in cultures of CHO cells in the presence of normal concentrations of fetal calf serum (10% FCS). Under these normal conditions, *reag* transfected cells grow several folds faster than untransfected cells (WT).

Figure 2 shows a comparable experiment at very low concentrations of fetal calf serum (0.5% FCS). These low serum concentrations do not allow wild-type cells to grow; after a few hours, the cells start to die. However, *reag* transfected cells are able to proliferate under the same conditions. The ability to overcome the growth arrest induced by the absence of growth factors is one of the typical properties of malignant transformation (cf Figure 18).

Not only the metabolic activity can be used to trace the proliferation in culture. The measurement of DNA synthesis is a more direct estimation of the rate of cell growth, since only cells entering S phase (committed to divide) synthesize DNA. Also DNA synthesis becomes serum-independent in *reag* transfected cells, i.e., the growth is maintained in the absence of growth factors (while it induces the programmed death of non-transfected cells). This is depicted in Figure 3, were the incorporation of 5-Bromo-2'-deoxyuridine[7-10] (BrdU) was used to monitor DNA synthesis in the presence of 10 or 0.5% FCS in CHO cells. As opposed to wild-type or cells transfected with an inactivating voltage-dependent potassium channel from rat brain (Kv1.4), there are no significant differences in the amount of DNA synthesized in the presence of normal or low FCS concentrations in *reag* -expressing cells. Similar experiments were done using epidermal growth factor (EGF) in HEK-293 cells or platelet-derived growth factor (PDGF) in CHO cells, with essentially the same result. The pure growth factors were used to avoid the complexity introduced by the use of whole serum.

To test the effects of *eag* on cell proliferation more directly, DNA synthesis was measured through incorporation of 5-Bromo-deoxyuridine (BrdU) in cells synchronized in the S-phase of the cell cycle by means of thymidine arrest (23). Consistent with the above mentioned findings, when the S-phase of the cell cycle was allowed to proceed, reag expressing CHO cells (CHOrEAG) showed higher metabolic activity (Fig 18B) and increased BrdU incorporation (Fig 18C). These results suggest that more *eag*-transfected cells entered the S-phase during the arrested period and/or DNA synthesis was elevated, in any case indicating a faster proliferation rate in CHOrEAG cells. In the presence of low serum, BrdU incorporation was significantly higher in CHOrEAG than in wild type cells (Fig 18C).

Yet another cell line, NIH3T3, has been frequently used for tumor transformation assays, since these cells are very strongly contact-inhibited, (i.e., their growth is stopped when the culture reaches confluency). This results in a homogeneous monolayer in wild-type cells. The malignant transformation of the line (through oncogene expression) usually induces the loss of this property, and NIH3T3 cells start forming colonies composed of several layers of cells. This can be seen after the transfection with r*eag* DNA, which induced the formation of such *foci* in several independent clones (Fig. 4A and B). Another standard test for transforming activity is the ability of NIH3T3 cells to grow in colonies when no substrate for attachment is available. To test this, cells are plated in an agar-containing medium, where the agar will prevent contacts between the cells and the surface of the plate. Under these conditions, wild-type NIH3T3 cells were unable to grow, while cells expressing r*eag* formed large colonies even detectable by simple visual inspection of the plate. Table I shows that r*eag*- (but not rKv1.4-) transfected cells formed colonies in a semisolid medium containing 0.3% agar (24,25), regardless of the vector used for transfection (Fig. 14). All of the above results indicate a transforming potential of *eag*.

Altogether, the results obtained from transfected cells indicate that r*eag* can, at least under certain conditions, display oncogenic properties.

Once the transforming ability of r*eag* was determined in accordance with the invention, the expression of the respective channel in human cancer cells was investigated. For this investigation, the cell line MCF-7 was used, which was initially obtained from a pleural effusion of a breast adenocarcinoma. The line is estrogen receptor positive as well as estrogen-sensitive and relatively well differentiated. The strategy followed was first to test electrophysiologically and pharmacologically for the presence of a functional current similar to *eag*, and then to try an identification of the corresponding channel at the molecular level. However, conventional approaches for such an identification failed.

Namely, in most cells, the current density was too low to allow reliable measurements of the whole cell current. Low current density precluded an accurate

measurement of channel properties using a whole cell configuration for the patch champ. Therefore, due to said low current densities encountered, another approach was resorted to. Due to such a low number of channels per cell, it is only possible to characterize the functional properties of a channel by a special patch champ method, excising patches of membranes containing one (or a few) channels and allowing characterization on a single molecule level. This approach relied on single-channel measurements in order to also compare properties at the single-molecule level such as single channel conductance, pharmacological properties, voltage dependence, and mean open times. Indeed, a channel with several properties compatible with r*eag* in terms of kinetics, voltage-dependence, and pharmacology in most membrane patches could thus be identified. Figure 5 shows whole-cell currents obtained from a MCF7 cell under nystatin patch conditions and single channel currents, together with their current-voltage relationship. Despite differences in kinetics at very depolarized voltages, the voltage dependence of the channel in human cells is highly reminiscent to the voltage-dependence of the r*eag* channel. Moreover, the single channel properties of the putative human-*eag* are also very similar to those of r*eag*.

Furthermore, standard approaches to isolate the said channel on a molecular level also were not successful. Several other groups have attempted and/or are still attempting to isolate the gene coding for a human *eag* without success and this in spite of the fact that the rat *eag* channel has already been published in 1994. For example, Warmke and Ganetzky (Proc. Natl. Acad. Sci. USA 91 (1994), 3428-3442) specifically set out to clone the human *eag* gene using conventional technology. They were, however, unsuccessful and cloned a novel, *eag* related gene which they termed *h-erg* (also referred to as *HERG*). Further, Wymore et al., Circulation Res. 80 (1997), 261-268, reported that no *eag* specific clones could be detected in a cDNA library from human heart in spite of the fact that primers for amplification were used that were conserved across the entire *eag/erg* superfamily. Thus, the standard approach with degenerated oligonucleotides based on the sequence of members of the family revealed itself unsuccessful, although *HERG* was systematically detected by other researchers in the field. Significantly, most of these approaches to clone the human *eag* gene were made with brain libraries. The conclusion from these combined prior art data was that the human *eag* gene could not be

cloned by conventional technology using the most obvious source, namely brain tissue. The repeated isolation of *HERG* clones instead is most probably due to the relative abundance of *HERG* transcripts in brain libraries, and also to the high homology between the two channels. Consequently, a different strategy had to be devised to direct the screening more specifically to *eag* channels. First, as described herein above, a cell line expressing a channel functionally similar to r*eag* was identified. Then degenerated oligonucleotides based on conserved sequences between rat, bovine and mouse *eag*, but divergent from *HERG* were designed. Using these primers, the cDNA obtained from MCF7 cells by PCR was amplified, and a band of the expected size was cloned in a suitable vector and sequenced. The amplified fragment corresponded to approximately 400 bp within the core region of the channel protein, and shared 90% identity to the r*eag* sequence at the DNA level, and 99% at the amino acid level. However, at this stage it was still quite unclear what the thus identified clone corresponded to. For example, it was quite possible that a further member of the *eag* family had been identified. This is in particular true in view of the fact that despite of a number of attempts with brain libraries, nobody had been able to clone the human *eag* gene and that the MCF7 line is a breast cancer derived line.

Since MCF7 cells are immortal cells, it is assumed that a number of genes is mutated. Ab initio, it could have been expected that the human *eag* channel, if at all expressed in this cell line, was mutated. Under this assumption, it was quite uncertain whether this cell line could at all be used for the isolation of the desired gene.

Due to the prior art failures to clone human *eag* gene from brain libraries and the above recited uncertainties with immortalized cell lines, another source for a library was in need. The 400 bp fragment was therefore used to screen a normal human breast cDNA library. Due to the presence of *eag* in breast cancer cells, such a library was expected to comprise h*eag* clones. Surprisingly, however, after screening 2x 10^6 phages, no human-*eag* clones could be identified in said library. This rises the possibility that the channel is expressed only in tumor cells, and not in normal tissue. Specific oligonucleotides, namely 5'-CCAAACACACACACCAGC, 5'-CGTGGATGTTATCTTTTTGG to check for h*eag* fragments by PCR amplification directly from the above library were designed,

but no evidence for the presence of any *eag* clones in this library was found. In view of the above discussed prior art results, it came as a further surprise that the same primers detected h*eag* in a normal human brain cDNA library, that was therefore screened. First, the probe obtained from MCF7 cells was used to check 10^6 phages. This procedure allowed to isolate a 1.6 kbp fragment from human *eag*. This fragment was then used as a probe for the screening of $2x10^6$ phages from the same library. Several independent clones were isolated, but none of them was a full-length clone. Furthermore, only one clone contained the 5' end of the sequence, while two of them contained the 3' end and part of the 3' non-coding region. It is likely that the abundance of restriction sites in the nucleic acid sequence encoding the channel has induced this extensive fragmentation of the cDNA. For example, when EcoRI was used to extract the inserts of the library that was cloned in λ-gt10 phage at the EcoRI site, this conventional approach systematically failed to find the 5' end of the molecule (there is an EcoRI site at position 400 of the clone). The pooled positive clones were therefore screened again by PCR, trying to amplify the start condon, and only by this means it was possible to isolate one phage that contained this ATG. Two splice variants of h*eag* were cloned, both expressed in brain tissue. The sequence obtained for h*eag* 1 and h*eag* 2 and their deduced amino acid sequence are shown in Figures 10 and 11, and compared to other members of the family.

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The deduced amino acid sequence is identical to the sequence published after the priority date of the present invention by Occhidoro (27) and is 97,7% identical to r*eag*. As mentioned, a second (81 bp longer) splice variant (h*eag* 2) was also isolated analogous to that reported for bovine and mouse *eag* channels (28), the splice insertion being identical in all three species. The chromosomal localization of h*eag* was determined by FISH detection (29) to map to chromosome lq32.1-32.3 (see also ref. 26).

To further check the possibility that h*eag* is not expressed in normal mammary gland, as opposed to MCF-7 cancer cells, we performed single-tube RT-PCR experiments using total RNA from human brain, human mammary gland, and MCF-7 cells (Fig 12), using as primers two oligonucleotides designed to discriminate between the two splice variants of h*eag*. In human brain, two splice variants were detected, while only the short one was expressed in MCF-7 cells (this, together with the lack of

amplification in the absence of reverse transcriptase, rules out a possible contamination by genomic DNA of the RNA preparation). No h*eag* signal was detected in normal mammary gland RNA with this highly sensitive technique. This result was totally unexpected, because preliminary results had suggested that expression was present in tumor cells from the same organ. Further, after Southern blot analysis of the RT-PCR products a faint band hybridizing with a h*eag* probe in mammary gland was identified. Accordingly, it is quite difficult to make a strong statement on the total absence of h*eag* message in breast in view of these contradictory experimental data.

Furthermore, electrophyiological properties (21, 30) of h*eag* were tested in *Xenopus* oocytes. As described above, they did not differ significantly from those or r*eag* with the above mentioned exceptions, e.g. a shift in activation of 40 mV to more depolarized potentials when both channels were measured under identical conditions. The electrophysiological observations of h*eag* channels expressed in *Xenopus* oocytes correlate well to hose reported by Bijlenga et al. (31).

The present invention also relates to a nucleic acid molecule specifically hybridizing to the nucleic acid molecule of the invention which comprises the sequence 5'-GGGAGGATGACCATGGCT.

This embodiment of the present invention is particularly useful for specific antisense therapies for inhibiting cell proliferation as will be discussed in more detail herein below (e.g. in Example 5). In addition, this embodiment of the nucleic acid molecule of the invention can, naturally, also be used as a probe for specifically detecting h*eag* mRNA in tissues, for example, by employing the Northern Blot technology. The analysis of h*eag* mRNA expression in various tissues by Northern blot revealed a strong hybridization signal of approximately 9.2 kb in brain and a weak signal of similar size in placenta. Heart, lung, liver, skeletal muscle, kidney and pancreas were negative even following long exposures. In addition, total RNA from human brain, heart, trachea, adrenal gland, liver, kidney, skeletal muscle and mammary gland, and spinal cord poly(A)$^+$ RNA, as well as total RNA from the adenovirus-transformed line 293 (a human non-tumoral cell line) were assayed by single-tube RT-PCR and Southern blot. Under

these experimental conditions, h*eag* was detected in brain only, where both splice variants were identified (Fig. 15; Example 3).

The preferential expression of h*eag* in brain was intriguing since the first cDNA had been isolated from an epithelial tumor cell line (MCF-7) and not from brain tissue (see above). To elucidate the presence of h*eag* in other tumoral cell lines, total RNA was prepared from HeLa (cervix carcinoma), SHSY-5Y (neuroblastoma), and lines from mammary gland tumors: COLO-824 (carcinoma), EFM-19 (carcinoma), and BT-474 (ductal carcinoma). Total RNA from brain, MCF-7 cells, 293 cells and RNA from cultures of mammary gland epithelial cells (included to circumvent the mixed cell populations in whole mammary gland) served as controls. All cell lines were obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) and maintained following the DSMZ catalog guidelines. Normal human mammary epithelial cells were purchased from BioWhittaker. The primers were designed to amplify different bands for h*eag* 1 and h*eag* 2, thus allowing us to rule out false positives due to genomic DNA contamination (controls in the absence of reverse transcriptase were also performed). HeLa, SHSY-5Y, EFM-19 and MCF-7 RNA exhibited an h*eag* band, whereas COLO-824 and BT-474 signals were indistinguishable from background (Fig. 15B). Cultured epithelial cells and 293 cells (Fig. 15A) were negative. As discussed above, it could be shown in accordance with the present invention that r*eag* transfected cells can display oncogenic properties. Thus, to determine whether the expression of h*eag* is advantageous for tumor cells *in vivo*, subcutaneous implants of CHO cells expressing the channel (CHOhEAG cells) into the flank of female scid (severe combined immunodeficiency, 32) mice were performed and it could be shown that expression of h*eag* represents an advantage for the proliferation of tumor cells *in vivo*, since CHOhEAG tumors grow faster and are more aggressive than CHOKv tumors.

Thus, the embodiment of the nucleic acid molecule of the present invention may be employed in the quantitative and qualitative analysis of the expression level of human *eag* in various disease states detectable in a tissue that may be indicative of, for example, cancer (in particular mamma carcinoma, neuroblastoma), psoriasis, neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, lateral amyotrophic sclerosis or multiple sclerosis.

In a preferred embodiment of the nucleic acid molecule of the invention, said nucleic acid molecule is DNA, such as genomic DNA. Whereas the present invention also comprises synthetic or semi-synthetic DNA molecules or derivatives thereof, such as peptide nucleic acid, the most preferred DNA molecule of the invention is cDNA.

In a further preferred embodiment of the present invention, said nucleic acid molecule is RNA, preferably mRNA.

Another preferred embodiment of the nucleic acid molecule of the invention encodes a fusion protein. For example, the nucleic acid molecule of the invention can be fused in frame to a detectable marker such as FLAG or GFP.

The invention further relates to a vector, particularly plasmid, cosmids, viruses and bacteriophages comprising the nucleic acid molecule of the invention. Such vectors may comprise further genes such as marker genes which allow for the selection of said vector in a suitable host cell and under suitable conditions. Thus the polynucleotide of the invention can be operatively linked in said vector to expression control sequences allowing expression in prokaryotic or eukaryotic cells. Expression of said polynucleotide comprises transcription of the polynucleotide into a translatable mRNA. Regulatory elements ensuring expression in eukaryotic cells, preferably mammalian cells, are well known to those skilled in the art. They usually comprise regulatory sequences ensuring initiation of transcription and optionally poly-A signals ensuring termination of transcription and stabilization of the transcript. Additional regulatory elements may include transcriptional as well as translational enhancers. Possible regulatory elements permitting expression in prokaryotic host cells comprise, e.g., the *lac*, *trp* or *tac* promoter in *E. coli*, and examples for regulatory elements permitting expression in eukaryotic host cells are the *AOX1* or *GAL1* promoter in yeast or the CMV-, SV40- , RSV-promoter (Rous sarcoma virus), CMV-enhancer, SV40-enhancer or a globin intron in mammalian and other animal cells. Beside elements which are responsible for the initiation of transcription such regulatory elements may also comprise transcription termination signals, such as the SV40-poly-A site or the tk-poly-A site, downstream of the

polynucleotide. In this context, suitable expression vectors are known in the art such as Okayama-Berg cDNA expression vector pcDV1 (Pharmacia), pCDM8, pRc/CMV, pcDNA1, pcDNA3 (In-vitrogene), pSPORT1 (GIBCO BRL).

Preferably, said vector is an expression vector and/or a gene transfer or targeting vector. Expression vectors and gene targeting or transfer vectors are well-known in the art and can be adapted for specific purposes of the invention by the person skilled in the art. Thus, expression vectors derived from viruses such as retroviruses, vaccinia virus, adeno-associated virus, herpes viruses, or bovine papilloma virus, may be used for delivery of the polynucleotides or vectors of the invention into targeted cell populations. Methods which are well known to those skilled in the art can be used to construct recombinant viral vectors; see, for example, the techniques described in Sambrook, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory (1989) N.Y. and Ausubel, Current Protocols in Molecular Biology, Green Publishing Associates and Wiley Interscience, N.Y. (1989). Alternatively, the polynucleotides and vectors of the invention can be reconstituted into liposomes for delivery to target cells.

The invention furthermore relates to a host transformed with the vector of the invention. Said host may be a prokaryotic or eukaryotic cell; see supra. The polynucleotide or vector of the invention which is present in the host cell may either be integrated into the genome of the host cell or it may be maintained extrachromosomally. In this respect, it is also to be understood that the recombinant DNA molecule of the invention can be used for "gene targeting" and/or "gene replacement", for restoring a mutant gene or for creating a mutant gene via homologous recombination; see for example Mouellic, Proc. Natl. Acad. Sci. USA, 87 (1990), 4712-4716; Joyner, Gene Targeting, A Practical Approach, Oxford University Press. Preferably, the host is a mammalian cell, a fungal cell, a plant cell, an insect cell or a bacterial cell. Preferred fungal cells are, for example, those of the genus Saccharomyces, in particular those of the species S. cerevisiae. The term "prokaryotic" is meant to include all bacteria which can be transformed or transfected with a polynucleotide for the expression of the protein of the present invention. Prokaryotic hosts may include gram negative as well as gram positive bacteria such as, for example, *E. coli*, *S. typhimurium*, *Serratia marcescens* and *Bacillus subtilis*. Methods for preparing fused, operably linked genes and expressing

them in bacteria or animal cells are well-known in the art (Maniatis, et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1989). The genetic constructs and methods described therein can be utilized for expression the protein of the present invention in prokaryotic hosts. In general, expression vectors containing promoter sequences which facilitate the efficient transcription of the inserted polynucleotide are used in connection with the host. The expression vector typically contains an origin of replication, a promoter, and a terminator, as well as specific genes which are capable of providing phenotypic selection of the transformed cells. The transformed prokaryotic hosts can be grown in fermentors and cultured according to techniques known in the art to achieve optimal cell growth. The polypeptides of the invention can then be isolated from the grown medium, cellular lysates, or cellular membrane fractions. The isolation and purification of the microbially or otherwise expressed polypeptides of the invention may be by any conventional means such as, for example, preparative chromatographic separations and immunological separations such as those involving the use of monoclonal or polyclonal antibodies. As regards mammalian cells, HEK 293, CHO, HeLa and NIH 3T3 are preferred. As regards insect cells, it is most preferred to use Spodoptera frugiperda cells, whereas the most preferred bacterial cells are E.coli cells.

The invention also relates to a method of producing the (poly)peptide encoded by the nucleic acid molecule of the invention comprising culturing the host of the invention and isolating the produced (poly)peptide.

Depending on the vector constructing employed, the (poly)peptide of the invention may be exported to the culture medium or maintained within the host. Suitable protocols for obtaining the (poly)peptide produced are well-known in the art for both ways of (poly)peptide production.

The present invention furthermore relates to a (poly)peptide encoded by the nucleic acid molecule of the invention or produced by the method of the invention. The new channel is envisaged to show a structure having a short amino-terminal region, probably intracellular, five membrane-spanning segments, a hydrophobic hairpin

entering the membrane, a sixth transmembrane segment, and a long C-terminal cytoplasmic part comprising a cyclic-nucleotide binding consensus sequence, a nuclear localization consensus sequence, and a hydrophobic domain probably forming a coiled-coil structure. The polypeptide of the invention may also be a functional fragment of the human K^+ ion channel. By "functional fragment" polypeptides are meant that exhibit any of the activity of h*eag* as described above. Using recombinant DNA technology, fragments of the (poly)peptide of the invention can be produced. These fragments can be tested for the desired function, for example, as indicated above, using a variety of assay systems such as those described in the present invention. Preferably, said fragments comprise the C-terminal portion of the novel ion channel.

The present invention also relates to an antibody specifically directed to the (poly)peptide of the invention. The antibody of the invention specifically discriminates between the human *eag* channel and the prior art channels such as mouse and rat *eag* and preferably binds to epitopes in the C-terminal part of the ion channel. The term "antibody", as used in accordance with the invention, also relates to antibody fragments or derivatives such as $F(ab)_2$, Fab', Fv or scFv fragments; see, for example, Harlow and Lane, "Antibodies, A Laboratory Manual", CSH Press 1988, Cold Spring Harbor, NY. Preferably, the antibody of the invention is a monoclonal antibody.

The invention also relates to a pharmaceutical composition comprising the nucleic acid molecule of the invention, the vector of the invention, the polypeptide of the invention and/or the antibody of the invention and a pharmaceutically acceptable carrier and/or diluent and/or excipient.

Examples of suitable pharmaceutical carriers and diluents as well as of excipients are well known in the art and include phosphate buffered saline solutions, water, emulsions, such as oil/water emulsions, various types of wetting agents, sterile solutions etc. Compositions comprising such carriers can be formulated by well known conventional methods. These pharmaceutical compositions can be administered to the patient in need thereof at a suitable dose. Administration of the suitable compositions may be effected by different ways, e.g., by oral, intravenous, intraperitoneal,

subcutaneous, intramuscular, topical or intradermal administration. The dosage regimen will be determined by the attending physician and clinical factors. As is well known in the medical arts, dosages for any one patient depend upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. Generally, the regimen as a regular administration of the pharmaceutical composition should be in the range of 1 μg to 10 mg units per day. If the regimen is a continuous infusion, it should also be in the range of 1 μg to 10 mg units per kilogram of body weight per minute, respectively. Progress can be monitored by periodic assessment. Dosages will vary but a preferred dosage for intravenous administration of DNA is from approximately 10^6 to 10^{12} copies of the DNA molecule. The compositions of the invention may be administered locally or systemically. Administration will generally be parenterally, e.g., intravenously; DNA may also be administered directly to the target site, e.g., by biolistic delivery to an internal or external target site or by catheter to a site in an artery.

It is envisaged by the present invention that the various polynucleotides and vectors of the invention are administered either alone or in any combination using standard vectors and/or gene delivery systems, and optionally together with a pharmaceutically acceptable carrier or excipient. Subsequent to administration, said polynucleotides or vectors may be stably integrated into the genome of the subject. On the other hand, viral vectors may be used which are specific for certain cells or tissues and persist in said cells or tissues. Suitable pharmaceutical carriers and excipients are, as has been stated above, well known in the art. The pharmaceutical compositions prepared according to the invention can be used for the prevention or treatment or delaying of different kinds of diseases, which are related to the undesired (over)expression of the above identified nucleic acid molecule of the invention. In a preferred embodiment the pharmaceutical composition comprises antisense oligodesoxynucleotides, as for example described in example 5, capable of regulating, preferably decreasing heavy expression.

Furthermore, it is possible to use a pharmaceutical composition of the invention which comprises the polynucleotide or vector of the invention in gene therapy. Suitable

gene delivery systems may include liposomes, receptor-mediated delivery systems, naked DNA, and viral vectors such as herpes viruses, retroviruses, adenoviruses, and adeno-associated viruses, among others. Gene therapy, which is based on introducing therapeutic genes, for example for vaccination into cells by ex-vivo or in-vivo techniques is one of the most important applications of gene transfer. Suitable vectors, methods or gene-delivery systems for in-vitro or in-vivo gene therapy are described in the literature and are known to the person skilled in the art; see, e.g., Giordano, Nature Medicine 2 (1996), 534-539; Schaper, Circ. Res. 79 81996), 911-919; Anderson, Science 256 (1992), 808-813; Isner, Lancet 348 (1996), 370-374; Muhlhauser, Circ. Res. 77 (1995), 1077-1086; Onodera, Blood 91 (1998), 30-36; Verzeletti, Hum. Gene Ther. 9 (1998), 2243-2251; Verma, Nature 389 (1997), 239-242; Anderson, Nature 392 (Supp. 1998), 25-30; Wang, Gene Therapy 4 (1997), 393-400; Wang, Nature Medicine 2 (1996), 714-716; WO 94/29469; WO 97/00957; US 5,580,859; US 5,589,466; US 4,394,448 or Schaper, Current Opinion in Biotechnology 7 (1996), 635-640, and references cited therein. The nucleic acid molecules and vectors of the invention may be designed for direct introduction or for introduction via liposomes, or viral vectors (e.g. adenoviral, retroviral) into the cell. Additionally, a baculoviral system can be used as eukaryotic expression system for the nucleic acid molecules of the invention. Delivery of nucleic acids to a specific site in the body for gene therapy may also be accomplished using a biolistic delivery system, such as that described by Williams (Proc. Natl. Acad. Sci. USA 88 (1991), 2726-2729).

Standard methods for transfecting cells with recombinant DNA are well known to those skilled in the art of molecular biology, see, e.g., WO 94/29469. Gene therapy may be carried out by directly administering the recombinant DNA molecule or vector of the invention to a patient or by transfecting cells with the polynucleotide or vector of the invention ex vivo and infusing the transfected cells into the patient. Furthermore, research pertaining to gene transfer into cells of the germ line is one of the fastest growing fields in reproductive biology. Gene therapy, which is based on introducing therapeutic genes into cells by ex vivo or in vivo techniques is one of the most important applications of gene transfer. Suitable vectors and methods for in vitro or in vivo gene therapy are described in the literature and are known to the person skilled in the art; see,

e.g., WO94/29469, WO 97/00957 or Schaper (Current Opinion in Biotechnology 7 (1996), 635-640) and references cited above. The polynucleotides and vectors comprised in the pharmaceutical composition of the invention may be designed for direct introduction or for introduction via liposomes, or viral vectors (e.g. adenoviral, retroviral) containing said recombinant DNA molecule into the cell. Preferably, said cell is a germ line cell, embryonic cell, stem cell or egg cell or derived therefrom. An embryonic cell can be for example an embryonic stem cell as described in, e.g., Nagy, Proc. Natl. Acad. Sci. USA 90 (1993) 8424-8428.

It is to be understood that the introduced polynucleotides and vectors of the invention express the (poly)peptide of the invention after introduction into said cell and preferably remain in this status during the lifetime of said cell. For example, cell lines which stably express the polynucleotide under the control of appropriate regulatory sequences may be engineered according to methods well known to those skilled in the art. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with the polynucleotide or vector of the invention and a selectable marker, either on the same or separate vectors. Following the introduction of foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective medium. The selectable marker in the recombinant plasmid confers resistance to the selection and allows for the selection of cells having stably integrated the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. Such engineered cell lines are particularly useful in screening methods or methods for identifying an inhibitor of the polypeptide of the present invention as described below.

A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, Cell 11(1977), 223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska, Proc. Natl. Acad. Sci. USA 48 (1962), 2026), and adenine phosphoribosyltransferase (Lowy, Cell 22 (1980), 817) in tk, hgprt or aprt cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler, Proc. Natl. Acad. Sci. USA 77 (1980), 3567; O'Hare, Proc. Natl. Acad. Sci. USA 78 (1981), 1527), gpt,

which confers resistance to mycophenolic acid (Mulligan, Proc. Natl. Acad. Sci. USA 78 (1981), 2072), neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, J. Mol. Biol. 150 (1981), 1), hygro, which confers resistance to hygromycin (Santerre, Gene 30 (1984), 147), Shble, which confers resistance to Zeocin® (Mulsant, Somat. Cell. Mol. Genet. 14 (1988), 243-252 or puromycin (pat, puromycin N-acetyl transferase). Additional selectable genes have been described, for example, trpB, which allows cells to utilize indole in place of tryptophan; hisD, which allows cells to utilize histinol in place of histidine (Hartman, Proc. Natl. Acad. Sci. USA 85 (1988), 8047); and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue, 1987, In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory ed.). Cells to be used for *ex vivo* gene therapy are well known to those skilled in the art. For example, such cells include for example cancer cells present in blood or in a tissue or preferably the corresponding stem cells.

Furthermore, the invention relates to a diagnostic composition comprising the nucleic acid molecule of the invention, the vector of the invention, the polypeptide of the invention and/or the antibody of the invention.

The diagnostic composition of the invention is useful in detecting the onset or progress of diseases related to the undesired expression or overexpression of the nucleic acid molecule of the invention. As has been pointed out herein above, such diseases are interrelated or caused by an increased or ongoing cellular proliferation. Accordingly, the diagnostic composition of the invention may be used for assessing the onset or the disease status of cancer. Having thus an early criterium for tumor activity, suitable counter-measures can immediately be applied. Such an immediate action will, of course, significantly improve the prognosis of the patient. These considerations equally apply to the diagnosis of metastases and recurrent tumors.

On the other hand, not all types of tumors may be characterized by an undesired expression or overexpression of the nucleic acid molecule of the invention. Alternatively, said (over)expression may occur only in certain stages, such as early stages, of tumor

development. Therefore, the diagnostic composition of the invention may also or alternatively be employed as a means for the classification of tumors or of the developmental status of a tumor. Naturally, the or most of the applications of the composition of the invention described here for tumors also apply to other diseases interrelated with or caused by the undesired (over)expression of the nucleic acid molecule of the invention.

Furthermore, a disease as recited throughout this specification also could be caused by a malfunction of the polypeptide of the present invention. Said disease could be interrelated or caused by, for example, an increased or reduced gene dosis of the polypeptide of the present invention, an increased or reduced activity of said polypeptide e.g. due to a modification in the primary amino acid sequence as compared to the corresponding wild-type polypeptide in a cell or tissue or a loss of the regulation of the activity of said polypeptide. Said disease might further be caused by an incorrect expression of the polypeptide during cell cycle progression or cell development. For example, mutated binding sites to intracellular or extracellular compounds, e.g. ions or second messengers or regulatory proteins, might result in a malfunction of the polypeptide of the present invention as it changes the binding characteristics for said compounds regulating the activity of said polypeptide. Malfunction could also be caused by defective modifications sites, for example, phosphorylation or glycosylation sites. It also might be caused by incorrect splicing events and therefore by expression of a truncated or extended polypeptides, for example, if h*eag* 1 is expressed instead of h*eagh* 2 or vice versa.

Thus, in a further embodiment the diagnostic composition described above could also be used to detect a malfunction of the polypeptide of the present invention.

In a further embodiment, the invention relates to a method for preventing or treating a disease which is caused by the malfunction of the polypeptide of the invention, comprising introducing an inhibitor of the expression of the nucleic acid molecule of the present invention or an inhibitor or a modifying agent of the malfunction of the (poly)peptide of the present invention or a nucleic acid molecule coding h*eag* or a polypeptide having h*eag* activity into a mammal affected by said disease or being

suspected of being susceptible to said disease. Methods for introduction of a nucleic acid molecule of the present invention encoding h*eag* into a cell or subject, i.e. gene therapy, are described within this specification as well as methods for the identification of inhibitors of the expression of a nucleic acid molecule of the present invention. Furthermore, inhibitors or modifying agents of the malfunction of the polypeptide of the present invention can be identified according to methods for the identification of inhibitors inhibitors of the polypeptide of the present invention known to a person skilled in the art (see below). For example, some genetic changes causing a malfunction of the polypeptide of the present invention lead to altered protein conformational states. Mutant proteins could possess a tertiary structure that renders them far less capable of fascilitating ion transport. Restoring the normal or regulated conformation of mutated proteins is the most elegant and specific means to correct these molecular defects. Pharmacological manipulations thus may aim at restoration of wild-type conformation of the protein. Thus, the polynucleotides and encoded proteins of the present invention may also be used to design and/or identify molecules which are capable of activating the wild-type function of a derivative of the polypeptide of the present invention displaying said malfunction.

The doses and routes for the administration for the treatment of a patient in need thereof have already been discussed herein above in connection with the pharmaceutical composition of the invention. Diseases that may be treated using the method of the present invention comprise any diseases that are correlated with cellular proliferation. Preferred diseases that fall into this category are tumor diseases such as cancer (breast cancer, neuroblastoma etc.), psoriasis, and degenerative diseases, especially those of the nervous system such as Alzheimer's disease, multiple sclerosis, lateral amyotrophic sclerosis, and Parkinson's disease.

Preferably, said inhibitor of the expression or overexpression of said nucleic acid molecule is the nucleic acid molecule of the invention that hybridizes to the nucleic acid molecule encoding the ion channel of the invention or fragment thereof. For example, this nucleic acid molecule can be an antisense oligodesoxynucleotide (ODN). The inventors could show that antisense ODNs treatment significantly reduces DNA

synthesis of several tumor cells, e.g. EFM cells, SHSY-5Y cells and HeLa cells (Example 5). Thus, in a preferred embodiment the nucleic acid molecule comprises antisense ODNs.

In a further preferred embodiment, said inhibitor of polypeptide function is the antibody of the invention or a drug. Said drug can be histamine receptor H1 inhibitor. Preferably, said drug inhibits active h*eag*, for example, acts as use-dependent, probably open-channel blocker, preferably said drug is astemizole or terfenadine. Further suitable drugs can be identified or designed by the person skilled in the art on the basis of the teachings of the present invention. Preferably, the drug will have an affinity to h*eag* channel in the mM range, more preferable in the nM range or lower. Preferably, the drug has no effect on other channels, for example on cardiac channels.

In a further preferred embodiment of the invention, said method further comprises prior to the introduction step,

(a) obtaining cells from the mammal infected by said disease and, after said introduction step, wherein said introduction is effected into said cells,

(b) reintroducing said cells into said mammal or into a mammal of the same species.

This embodiment of the present invention is particularly useful for gene therapy purposes which will reduce the treatment duration largely and increase the effectivity and reduce (even eliminate) side effects. In addition, this embodiment of the method of the invention can also be employed in the context or in combination with conventional medical therapy. The removal from and the reintroduction into said mammal may be carried out according to standard procedures.

Preferably, the above referenced cell is a germ cell, an embryonic cell or an egg cell or a cell derived from any of these cells.

The invention further relates to a method of designing a drug for the treatment of a disease which is caused by the undesired expression or overexpression of the nucleic acid molecule of the invention comprising:

(a) identification of a specific and potent drug;

(b) identification of the binding site of said drug by site-directed mutagenesis and chimeric protein studies;

(c) molecular modeling of both the binding site in the (poly)peptide and the structure of said drug; and

(d) modifications of the drug to improve its binding specificity for the (poly)peptide.

The term "specific and potent drug" as used herein refers to a drug that potently and specifically blocks h*eag* function.

All techniques employed in the various steps of the method of the invention are conventional or can be derived by the person skilled in the art from conventional techniques without further ado. Thus, biological assays based on the herein identified features of the ion channel of the invention may be employed to assess the specificity or potency of the drugs wherein the decrease of one or more activities of the ion channel may be used to monitor said specificity or potency. Steps (b) and (d) can be carried out according to conventional protocols described, for example, in K.L. Choi, C. Mossman, J. Aubé & G. Yellen. The International Quaternary Ammonium Receptor Site of *Shaker* Potassium Channels. Neuron 10, 533-541 (1993), C.-C. Shieh & G.E. Kirsch: Mutational Analysis of Ion Conduction and Drug Binding Sites in the Inner Mouth of Voltage-Gated K^+-Channels. Biophys. J. 67, 2316-2325 (1994), or C. Miller: The Charybdotoxin Family of K^+-Channel-Blocking Peptide. Neuron 15, 5-10 (1995).

For example, identification of the binding site of said drug by site-directed mutagenesis and chimerical protein studies can be achieved by modifications in the (poly)peptide primary sequence that affect the drug affinity; this usually allows to precisely map the binding pocket for the drug.

As regards step (c), the following protocols may be envisaged: Once the effector site for drugs has been mapped, the precise residues interacting with different parts of the

drug can be identified by combination of the information obtained from mutagenesis studies (step (b)) and computer simulations of the structure of the binding site (since a potassium channel has recently been crystallized in the art, this can now be done by the person skilled in the art without further ado) provided that the precise three-dimensional structure of the drug is known (if not, it can be predicted by computational simulation). If said drug is itself a peptide, it can be also mutated to determine which residues interact with other in the h*eag* molecule.

Finally, in step (d) the drug can be modified to improve its binding affinity or its potency and specificity. If, for instance, there are electrostatic interactions between a particular residue of h*eag* and some region of the drug molecule, the overall charge in that region can be modified to increase that particular interaction; additionally, if those interactions occur with a region of h*eag* that is not conserved with other channel proteins, it is conceivable that an improvement of that interaction while other binding factors are weakened will improve the specificity of the drug.

Identification of binding sites may be assisted by computer programs. Thus, appropriate computer programs can be used for the identification of interactive sites of a putative inhibitor and the polypeptide of the invention by computer assisted searches for complementary structural motifs (Fassina, Immunomethods 5 (1994), 114-120). Further appropriate computer systems for the computer aided design of protein and peptides are described in the prior art, for example, in Berry, Biochem. Soc. Trans. 22 (1994), 1033-1036; Wodak, Ann. N. Y. Acad. Sci. 501 (1987), 1-13; Pabo, Biochemistry 25 (1986), 5987-5991. Modifications of the drug can be produced, for example, by peptidomimetics and other inhibitors can also be identified by the synthesis of peptidomimetic combinatorial libraries through successive chemical modification and testing the resulting compounds. Methods for the generation and use of peptidomimetic combinatorial libraries are described in the prior art, for example in Ostresh, Methods in Enzymology 267 (1996), 220-234 and Dorner, Bioorg. Med. Chem. 4 (1996), 709-715. Furthermore, the three-dimensional and/or crystallographic structure of inhibitors of the polypeptide of the invention can be used for the design of peptidomimetic inhibitors, e.g., in combination with the (poly)peptide of the invention (Rose, Biochemistry 35 (1996), 12933-12944; Rutenber, Bioorg. Med. Chem. 4 (1996), 1545-1558).

An exemplary strategy for identifying a specific inhibitor that may be used in accordance with the present invention is provided in the appended examples.

The invention also relates to a method of identifying an inhibitor of the expression of the nucleic acid of the invention or of a function of the (poly)peptide of the invention comprising:

(a) testing a compound for the inhibition or reduction of translation wherein said compound is selected from antisense oligonucleotides and ribozymes; or

(b) testing a compound for the inhibition of transcription wherein said compound binds to the promoter region of the gene encoding the (poly)peptide of the invention and preferably with transcription factor responsive elements thereof; or

(c) testing peptides or antibodies suspected to block the proliferative activity of the (poly)peptide of the invention for said blocking activity.

As regards alternative (b) referred to above, it may be advantageous to first characterize the promoter region and locate transcription factor responsive sequences in it. Then it would be possible to genetically manipulate the promoter to render it more sensitive to repressors or less sensitive to enhancers. Turning now to alternative (c), it may be advantageous to first locate the part or parts of the ion channel of the invention implicated in the generation of proliferation disorders. Compounds that have been positive in one of the test systems are, prima facie, useful as inhibitors.

Peptidomimetics, phage display and combinatorial library techniques are well-known in the art and can be applied by the person skilled in the art without further ado to the improvement of the drug or inhibitor that is identified by the basic method referred to herein above.

In a further embodiment, the present invention relates to a method of inhibiting cell proliferation comprising applying an inhibitor to expression of the nucleic acid of the invention or the (poly)peptide of the invention. The method of the invention may be carried out in vitro, ex vivo or when application is to a subject, in vivo.

The present invention also relates to a method of prognosing cancer and/or neurodegenerative diseases and/or psoriasis comprising assessing the expression of the nucleic acid molecule of the invention or assessing the quantitative presence of the (poly)peptide of the invention. In a preferred embodiment said cancer is a mamma carcinoma or neuroblastoma, in a more preferred embodiment said cancer is breast adenocarcinoma, breast carcinoma ductal type, or cervix carcinoma. In a further embodiment said neurodegenerative diseases is Alzheimer's disease, Parkinson's disease, lateral amytrophic sclerosis or multiple sclerosis.

The method of the invention may be carried out in vitro, in vivo, or ex vivo. Suitable protocols for carrying out the method of the invention are well-known in the art and include, as regards in vitro techniques, Northern blotting for the assessment of the level of mRNA or the analysis of tissue by microscopic techniques using, for example, antibodies that specifically recognize the (poly)peptide of the invention. One or more these techniques may be combined with PCR based techniques which may also or in combination with further (conventional) techniques be used for the above recited assessment.

In a preferred embodiment of the above-mentioned methods of the invention, said mammal is a human, rat or mouse.

The present invention further relates to the use of the nucleic acid molecules of the invention in gene therapy. As has been pointed out here above, gene therapy may be designed to inhibit cell proliferation and thus treat any disease affected thereby such as cancer or psoriasis in a specific way. The invention particularly envisages two independent lines carrying out such gene therapy protocols:

(a) Mutagenesis of the channel together with chemical engineering of H1 antagonists (preferably of astemizole) in order to obtain a drug specific for human *eag*;

(b) Quantitative and qualitative analysis of the expression levels of *eag* in cancer tissue, in order to design a diagnostic and/or prognostic method. This would also allow the design of genetic therapies against specific tumors.

For example, the nucleic acid molecule may be introduced in vivo into cells using a retroviral vector (Naldini et al., Science 272 (1996), 263-267; Mulligan, Science 260 (1993), 926-932) or another appropriate vector. Likewise, in accordance with the present invention cells from a patient can be isolated, modified in vitro using standard tissue culture techniques and reintroduced into the patient. Such methods comprise gene therapy or gene transfer methods which have been referred to herein above.

Finally, the present invention relates to a kit comprising the nucleic acid molecule specifically hybridizing to the nucleic acid molecule encoding the (poly)peptide of the invention, the vector of the invention, the polypeptide of the invention and/or the antibody of the invention.

The kit of the invention can, inter alia, be employed in a number of diagnostic methods referred to above. The kit of the invention may contain further ingredients such as selection markers and components for selective media suitable for the generation of transformed host cells and transgenic plant cells, plant tissue or plants. Furthermore, the kit may include buffers and substrates for reporter genes that may be present in the recombinant gene or vector of the invention. The kit of the invention may advantageously be used for carrying out the method of the invention and could be, inter alia, employed in a variety of applications referred to herein, e.g., in the diagnostic field or as research tool. The parts of the kit of the invention can be packaged individually in vials or in combination in containers or multicontainer units. Manufacture of the kit follows preferably standard procedures which are known to the person skilled in the art.

Several documents are cited throughout the text of this specification. Each of the documents cited herein (including any manufacturer's specifications, instructions, etc.) are hereby incorporated herein by reference; however, there is no admission that any document cited is indeed prior art as to the present invention.

The figures show:

Figure 1. Proliferation of wild-type (circles) and *reag*-expressing CHO cells as a function of time. Cells were plated in 96-well dishes and at the indicated times the tetrazolium salt MTT[6] (50 μg/ml) was added to the plates. After four hours incubation in humidified atmosphere (37 C°, 5%CO_2), the reaction was stopped by addition of 2 volumes of 10% SDS in 1M HCl. The blue formazan crystals produced in living cells were solubilized overnight, and the resulting color was measured as optical density at the indicated wavelength. Possible non-specific effects of the transfection on the cell proliferation can be neglected, since a) the results were comparable in three independent cell lines from different species (rat, hamster and human); b) transfection with different independent clones gave the same results, and c) transfection with a different potassium channel (Kv1.4) in the same vector (thus with a tendency to recombine at the same site) gave results comparable to WT and did not reproduce the effects of the *reag* transfection.

Figure 2. Proliferation of wild type (circles) and *reag* expressing (triangles) CHO cells, in the presence of 0.5% FCS. This serum concentration is not able to sustain growth of normal cells, but transfected cells complete almost three cycles. Methods as for Figure 1.

Figure 3. DNA synthesis in CHO cells expressing different potassium channels, in the presence of normal (10%) or low (0.5%) concentrations of FCS. In control cells, WT or cells transfected with Kv1.4, the levels of DNA synthesis drop significantly in the presence of low serum concentration, whereas *reag* expressing cells maintain the same replication levels as in high serum concentrations.

Figure 4. (A) Photographs of plates with wild type, Kv1.4 transfected or *reag* transfected NIH3T3 cells. The cells were seeded at low density, and allowed to grow under standard conditions until wild-type cells reached confluence. The cells were then fixed with methanol and stained with Giemsa blue. Under those conditions, both wild type and Kv1.4-expressing cells grow in a monolayer, whereas *reag* expressing cells form *foci*. (B) Foci formation of *reag* -transfected NIH-3T3 cells compared to cells transfected with rKv1.4 and to wild type cells. The vector control (pcDNA3 transfected cells) yielded a similar phenotype as wild type cells (not shown). Transient transfection was carried out

using calcium phosphate (33). Cells were maintained in rich medium until control cells reached confluence, then fixed with methanol and stained with Giemsa blue.

Figure 5. Currents elicited by depolarizations in MCF7 cells under voltage clamp conditions. Left traces are whole cell currents, right traces have been obtained in an excised outside-out patch. Both the macroscopic currents and the I-V relationships (C and D) are reminiscent of *reag* currents.

Figure 6. Single channel activity in an outside-out membrane patch voltage-clamped at 0 mV, in the presence or the absence of 5 μM astemizole. The pipette solution contained 140 mM KCl, 10 mM BAPTA, 10 mM HEPES pH 7.2; the bath solution contained 140 mM NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 2.5 mM KCl, 10 HEPES pH 7.2.

Figure 7. A. DNA synthesis in MCF7 cells under different *eag* blockers. B. HEK293 DNA synthesis levels in the presence of astemizole, glibenclamide and terfenadine.

Figure 8. Dose-response curve for the effects of two H1 antagonists on DNA synthesis in MCF7 cells (IC50 7 and 10 mM for LY 91241 and astemizole respectively).

Figure 9. Fluorescence images of control (untreated, A) and astemizole-treated (B) MCF7 cells, stained with Hoechst 33342. Notice in B the smaller surface of the nuclei, and a much lower cell density (due to cell death).

Figure 10. Nucleotide sequence of human-*eag* cDNA from human brain compared to the rat sequence and bovine sequences. Those positions showing a different nucleotide in any of the sequences are shaded.

Figure 11. Amino acid sequences of both splice variants obtained from human *eag* cDNA translation, compared to the corresponding bovine, mouse and rat sequences. The black boxes indicate a different residue in any of the sequences.

Figure 12. RT-PCR from human brain, human mammary gland and MCF-7 cells total RNA. The amplification produced two specific fragments corresponding to the expected sizes for h*eag* 1 and 2 in brain, and the band corresponding to h*eag* 1 in MCF-7 cells, while no amplification was detected in normal breast RNA.

Figure 13. Voltage-dependence of activation in high extracellular potassium, two-electrode voltage-clamp: In the conductance-voltage plot, the voltage for half-activation is shifted by 40 mV to the right in the h*eag* channel with respect to the r*eag* channel.

Figure 14. Colony formation in semisolid medium of NIH-3T3 cells transfected with the indicated DNAs. Cells were plated in regular medium containing 0.3% agar onto a layer of 0.55% agar medium. Colonies larger than 0.1 mm in diameter were scored 14 days after transfection. The average number of colonies in at least ten counted microscope fields is expressed per μg DNA used in the transfection (except for the lanes "Transfection buffer" and "No treatment", where the numbers are absolute values). r*eag* and Kv1.4 were transfected using either pcDNA3 or pTracer CMV vectors.

Figure 15. (A) Southern blot of RT-PCR products of RNAs from different human tissues and 293 cells. Transferrin receptor (TFR) signals are shown at the bottom. (B) Southern blot analysis of RT-PCR products of total RNAs from different human cell lines and mammary epithelial cells in primary culture (Epith. cells). TRF signals are shown at the bottom.

Figure 16. (A) Treatment of h*eag* expressing tumor cell lines with antisense ODNs. (B) h*eag* current in SHSY-5Y neuroblastoma cells (C) Current density in SHSY-5Y cells treated with antisense ODNs (D) Inhibition of DNA synthesis in human cancer cells (EFM-19, HeLa and SHSY-5Y) by antisense ODNs directed against h*eag*.

Figure 17. (A) Subcutaneous implantation of CHOhEAG cells induced aggressive tumors that grew rapidly and soon broke the skin of the carrier mice. The photograph was taken in the third week post-implantation of $2x10^6$ cells. (B,C) The average mass of CHOhEAG tumors was significantly greater than that of the CHOKv tumors both two

weeks (B; mean±S.E.M.; p=0.002) or three weeks post-implantation (C; mean±S.E.M.; p=0.03) (D) CHOhEAG and (E) CHOKv tumors photographed *in situ*. The main macroscopic differences are the darker color and the fixation to the skin of the CHOhEAG tumor. (F, G) CHOhEAG (F) and CHOKv (G) tumors were cut open to show the great extent of necrosis (arrowheads) in the former. (H, I) The greater degree of necrosis and the fixation to the skin are also evident microscopically after paraffin embedding and hematoxylin-eosin staining. The histology is comparable in both micrographs, but in (H) a much bigger necrotic area is observed (arrowheads), and there is no border between the subcutaneous fat and the tumor. (Scale bars, 100 μm) (J) As a quantitative measurement of these images, the average width of the vital area in CHOKv tumors was significantly larger than that of CHOhEAG tumors (mean±S.E.M.; $p<0.0005$).

Figure 18: Proliferation assays of rEAG-transfected CHO cells (A-C). Growth curves of CHO cells transfected with rEAG (circles) as compared to naive cells (triangles) in 10% (filled symbols) or 0.5% (open symbols) fetal calf serum. The values are referred to the ones measured after 12 h in culture (time 0 in the plot), and represent mean±S.E.M. of eight wells in the same plate. Cell lines were established by selection through the G-418 resistance encoded in the pcDNA3 vector. MTT hydrolysis (22) was used to measure metabolic activity of viable cells. Serum was carefully diluted 12 hours after plating. (B) Increase in metabolic activity during the first 12 hours after removal of S-phase block. For cell synchronization, 2 mM thymidine was added to the culture medium for 12 h. Thymidine was removed from the medium for additional 12 h, and then a second arresting pulse was applied for 12 h. Cells were then trypsinized and plated for metabolic activity and DNA synthesis determination. (C) BrdU incorporation during the first 12 hours after removal of S-phase block for 12h incubation in 10% FCS, or in the presence of 0.5% FCS (24 h incubation). BrdU incorporation was measured using the Boehringer-Mannheim "BrdU labeling and detection kit", following the indications of the manufacturer. The bars represent mean±S.D. for wild-type CHO cells (open bars), Kv1.4-transfected (shaded bars) and *eag*-transfected (solid bars). The incorporation of BrdU is quantified as optical density at 405 nm (reference 490 nm) produced on ABTS™ substrate by peroxidase coupled to the anti BrdU antibody.

The examples illustrate the invention.

Example 1: Cloning of the K^+ ion channel

mRNA was purified from total RNA obtained from MCF-7 cells following standard procedures. Then, cDNA was prepared by reverse transcription with Superscript II reverse transcriptase; this cDNA was used as a template for PCR amplification using degenerate oligonucleotides designed to match highly conserved *eag* sequences. After amplification, a SacII/SacII fragment from rat *eag* was used as a probe for Southern blot analysis of the results. Those bands showing positive hybridization were subsequently cloned in pGEM-T vector (Promega) and sequenced. All of them gave sequences corresponding to HERG.

Specific oligonucleotides engineered to avoid HERG cDNA amplification were then designed, taking into account rat, mouse and bovine *eag*. We looked for sequences having high homology between the various *eag* clones but with maximal divergence to the HERG sequence.

The sequences of the oligonucleotides were the following:

5'-CAGAA(T,C)AA(T,C)GTGGC(A,C,T,G,)TGGCT
5'-TCACT(G,A)AAGATCTATA(A,G)TC

After PCR amplification, the band of the expected size was cloned into pGEMT and sequenced. The sequence obtained showed high homology to rat *eag* (nucleotides 942-1108).

This band was labeled and used as a probe to screen a mammary gland cDNA library. After screening of $2x10^6$ phages, no positive clones were found.

We then used specific oligonucleotides to analyze cDNA using PCR from human heart and human brain (obtained from total RNA purchased from Clontech). Two PCR products from brain were sequenced, and the sequence corresponded to two alternatively spliced variants of *eag*. To further test the possibility of cloning the full length molecule from the human brain, we performed PCR analysis of a human cDNA library, and compared this result to the same experiment in the human mammary gland library (both from Clontech). Only the brain library gave positive results.

Subsequently, the amplified fragment was employed to screen the human brain library (2 rounds, 10^6 phages) and several clones that were cloned into the pBSK-vector were found and sequenced. All of them corresponded to the central part of the molecule, but were missing the 5' and 3' ends. The longest of these positive clones was used to prepare a probe and re-screen the library (again two rounds, $2x10^6$ clones).

The sequences obtained in this case corresponded to part of the coding sequence (approximately 400 bp 5' were missing until the initiation codon) and a long 3' untranslated sequence. Since the fragment close to the 5' end of the molecule started in all cases with an EcoRI site, it was suspected that the site was actually present in the h*eag* sequence, and that is was lost in the subcloning of the fragments into vectors for sequencing.

To obtain the full length sequence, we pooled those phages that carried fragments close to the 5' end and analyzed them by PCR amplification, using the sequence 3' to the mentioned EcoRI site and a sequence from lambda gt10 as primers for the PCR. After successive fractionation of the pools, two phages that carried the 5' end of the coding sequence were obtained, and one of them contained part of the 5' untranslated region.

Once we knew the complete sequence, we assembled the whole clone starting from two phages, one of them containing the 3' UTR and most of the coding sequence, and the other containing the 5' end. The first fragment was extracted from the phage by SphI/HindIII digestion, and subcloned into pBKS- to produce pBKSh*eag* 1. In this was, a 1.2 kbp SphI-SphI fragment was also removed from the clone, and it was necessary to

reintroduce it afterwards. The fragment containing the 5' end was extracted by HindIII/MunI digestion. This fragment was ligated with a HindIII/MunI digest of pBKSh*eag* 1. Only using this procedure were we able to obtain the full length clone in a single plasmid. We then needed to reintroduce the SphI-SphI fragment since we had deleted one of the SphI sites. Subsequently, an EagI/NotI fragment was subcloned into the NotI site of pCDNA3 vector, to eliminate the contaminating phage sequences and to obtain a vector suitable for functional expression of the channel. Finally obtained sequences are depicted in sequence listing as SEQ ID No. 1 and SEQ ID No. 2.

Example 2: Identification of inhibitors that specifically bloc the action of human *eag*.

Another member of the *eag* family, *HERG*[11-16], has been related to a familiar form of long QT syndrome (LQT). This has allowed to identify several blockers of *HERG* based on their ability to induce LQT-type arrythmias. Thus, certain histamine H1 receptor blockers, such as astemizole and terfenadine, as well as class III antiarrythmic drugs (dofetilide, E-4031) are potent and specific blockers of *HERG*[15,17]. However, for *eag* channels, specific blockers have not yet been described. Due to the sequence similarity between *HERG* and *eag* channels, both groups of drugs on r*eag* were tested in accordance with the present invention. The H1 blockers also affect r*eag*, whereas the channel is rather insensitive to class III antiarrythmics (dofetilide). This provides a useful tool to selectively block *eag*-type channels and to discard possible effects of *HERG* channels (which are also present in MCF7 cells). The effect of one of these drugs (astemizole 5 μM) is shown on single putative human *eag* channels in Fig. 6.

It was further tested whether several r*eag* and other potassium channel blockers are able to inhibit growth of MCF7 cells. As a "positive" control glibenclamide, a blocker of the ATP-sensitive potassium channel was also included, since it has been described to inhibit the proliferation of this cell line[2]. To determine the rate of DNA synthesis, cells were plated on 96-well microtiter plates at a density of $\approx 10^5$ cells/ml and in the absence of growth factors. After 24 hours starvation, cells were stimulated by addition of 10% FCS in the presence of BrdU. The amount of BrdU incorporated into the

newly synthesized DNA was determined using a commercial antibody (Boehringer Mannheim). The drugs tested were added either at the same time or 12 hours prior to the stimulation. In a different human cell line, HEK293, the addition of 10 μM astemizole or 100 μM glibenclamide did not reduce significantly the DNA synthesis, while terfenadine (10 μM) produced a strong inhibition. For this reason, only effects of astemizole (and its closely related analog LY91241) were considered, and those produced by terfenadine (although MCF7 cells are significantly more sensitive to growth inhibition by terfenadine than the control cells) discarded. In MCF7 cells, 5 μM astemizole reduced the DNA synthesis by 40%, while the same concentration of the *HERG*-specific blocker dofetilide produced no significant effects. Ten times higher concentrations (50 μM) of other potassium channel blockers (quinidine or glibenclamide) where required to induce a similar effect. A dose-response curve for astemizole effects on DNA synthesis in MCF7 cells is depicted in Figure 8. The half-maximal effect was obtained for 10 μM astemizole.

In an attempt to clarify the mechanism underlying the proliferation inhibition in MCF7 cells, the nuclear morphology of cells treated with 5 μM astemizole were checked, using the supravital nuclear stain Hoechst 33342. After 24 hours of treatment, most cells showed nuclear condensation and fragmentation, typical features of apoptotic cell death (Fig. 9).

In conclusion, a human counterpart of the *reag* channels are present in human cancer cells, and they have the ability to induce malignant transformation in several different cell types.

Example 3: Expression of h*eag* in different human tissues

500 ng total RNA from different human tissues (or 5 ng polyA$^+$ RNA, for spinal chord) were reverse transcribed and amplified using a pair of oligonucleotides of the sequences 5'-CGCATGAACTACCTGAAGACG (forward) and 5'-TCTGTGGATGGGGCGATGTTC (reverse). The amplified DNA was analyzed by Southern blot using a specific human *eag* probes (a 1.5 Kb EcoRI fragment from the core

of the channel). Among the RNAs tested, only brain total RNA gave positive signals. RNAs from spinal chord, adrenal gland, skeletal muscle, heart, trachea, liver, kidney and mammary gland were negative. The integrity of the RNA was checked using transferrin amplification. Using the same approach, the expression of h*eag* in several tumoral human cell lines was checked, in: MCF-7 (breast adenocarcinoma), BT-474 (breast ductal carcinoma, from a solid tumor), EFM-19 (breast carcinoma, ductal type, from pleural fluid), COLO-824 (breast carcinoma, from pleural fluid), SHSY5Y (neuroblastoma).

In contrast to normal tissues, all the cancer cell lines tested were found positive for h*eag* expression.

Further, Southern blot of RT-PCR products of RNAs from different human tissues and 293 cells show that only in RNA from brain the two bands corresponding to h*eag* A and B could be amplified and identified. Transferrin receptor (TFR) signals are shown at the bottom (Fig. 15A). Furthermore, a Southern blot analysis of RT-PCR products of total RNAs from different human cell lines and mammary epithelial cells in primary culture (Epith. cells). TRF signals are shown at the bottom. RNAs from the different cell lines (34) and commercial RNAs from human tissues (Clontech) were subjected to single-tube RT-PCR (35). Total RNA was used with the exception of spinal cord, where poly(A)$^+$ RNA was used (Primer sequences were: forward: 5'-CGCATGAACTACTGAAGACG, and reverse: 5'-TCTGTGGATGGGGCGATGTTC. 5'-TCAGCCCAGCAGAAGCATTAT and reverse: 5'-CTGGCAGCGTGTGAGAGC were used to control RNA and PCR performance.). Specific primers for TFR were used to control RNA and PCR performance. These ODNs were designed according to the published TFR sequence (36), starting at exon 11 and spanning to exon 19 (37). This, together with the amplification of two h*eag* splice fragments and controls in the absence of reverse transcriptase, excludes a false positive due to genomic DNA contamination. 50 µl (h*eag*) or 15 µl (TFR) of PCR reactions were analyzed in 2% agarose gels. DNA was transferred to membranes and consecutively hybridized at high stringency with [^{32}P]-dCTP labeled random primed probes consisting of a 980 bp h*eag* fragment and the TFR fragment amplified from brain RNA.

Example 4: Expression of *heag* in vivo

To determine whether the expression of h*eag* is advantageous for tumor cells *in vivo*, the inventors preformed subcutaneous implants of CHO cells expressing the channel (CHOhEAG cells) into the flank of female scid (severe combined immunodeficiency, 33) mice. CHOKv cells were used as a control. Therefore, $2x10^6$ CHOhEAG or CHO-Kv1.4 cells suspended in 100 µl PBS were implanted subcutaneously on the flank of 6-8 week old female Fox Chase scid mice (*C.B-17/Icr sicd/scid*) obtained from Bomholtgard, Ry, Denmark. The presence of tumors was checked every second day by tactile inspection of every mouse. After two or three weeks, the animals were sacrificed by cervical dislocation and the tumors dissected and fixed in paraformaldehyde for subsequent paraffin inclusion and staining. The identity of the CHOhEAG cells was established by UV illumination of the tumors to evoke fluorescence from the green fluorescence protein encoded in the pTracer vector (Invitrogen). One week after the implantation, all CHOhEAG-injected mice carried tumors detectable by palpation, while no mass greater than 1 mm was observed in the controls. During the second week post-implantation, the h*eag* -expressing tumors reached in excess of 5 mm in diameter and visibly emerged through the skin in most cases (Fig. 17A); the mice were sacrificed after two (N=6) or three weeks (N=7). Only one of the 11 control animals used was free of visible tumors; all 13 CHOhEAG-injected animals showed tumors. The average mass (Fig. 17B, C) of the h*eag* -expressing tumors was significantly larger than that of controls, especially two weeks following implantation (Fig. 17B). From macroscopic observation, the tumors appeared friable and hemorragic; the CHOhEAG tumors were darker than the controls and were adhered to the skin (Fig. 17D, E) in all CHOhEAG-injected mice at two weeks. Six of seven mice exhibited similar characteristics at three weeks. In contrast, the tumor could be easily dissected from the skin inall of the control mice after two weeks, and in five out of six mice at three weeks. The tissue below the tumor appeared unaffected in all cases. The dark color was due to great extent of intratumoral necrosis (Fig. 17 F, G, arrows), confirmed by histology (Fig. 17 H, I, arrowheads), indicating a faster growth of CHOhEAG tumors. The thickness of the vital area in the EAG-expressing tumors was significantly smaller than in the controls (Fig. 17J). The rapid growth of the tumor can account for the massive intratumoral necrosis in the CHOhEAG group. This could also

explain the enhanced difference found in the mass of the tumors two weeks after implantation, since CHOhEAG tumors would cease growth due to massive necrosis. These data strongly suggest that expression of h*eag* tumors grow faster and are more aggressive than CHOKv tumors.

Example 5: Inhibition of h*eag*

It is assumed that expression of h*eag* in some tumor cells is not the consequence of their abnormal growth, but that this K^+ channel is necessary for their proliferation. Therefore, inhibition of h*eag* expression with antisense oligodeoxynucleotides (ODNs) should decrease the proliferation rate in these tumor cells. Therefore, a 19-mer antisense phosphorothioate ODN (5'-CAGCCATGGTCATCCTCCC) spanning the putative initiation codon of h*eag* was used to test inhibition of proliferation. The sense ODN and a scrambled sequence (gtcggtaccagtaggaggg) were used as controls. Data shown in Figure 16A confirms the efficiency of the antisense ODN treatment in reducing the h*eag* mRNA content in EFM cells. A reduction in h*eag* mediated K^+ currents in SHSY-5Y cells by treatment with antisense ODN is shown in Fig. 16B and C.

Treatment of h*eag* expressing tumor cell lines with antisense ODNs significantly reduced the yield of amplified PCR products. EFM-19 cells were treated with 10 µg/ml DAC30 (lanes "C") or 10 µg/ml DAC30 (Eurogentec) plus 1 µM antisense ODN (lanes "AS") overnight, total RNA was extracted and assayed under the same conditions as described in Example 3, with ODNs designed to either amplify h*eag* or the transferrin receptor. The arrows in Fig. 16A mark the expected sizes of the amplified fragments. Further, to dissect the h*eag* current in SHSY-5Y neuroblastoma cells, the inventors utilized the voltage-dependence of the activation of *eag* (30) in the presence of extracellular Mg^{2+}. The current was measured after a depolarization to +60 mV from -120 mV (Fig. 16B, gray lines). The first part of the subtracted trace (Fig. 16B, black line) corresponds to *eag* current that has not yet activated when the holding potential is very negative (-120 mV), but becomes evident if the holding potential is -60 mV. The average current between 19 and 21 ms was chosen to determine the current density. The current density in SHSY-5Y cells treated with antisense ODNs was significantly reduced as compared to control cells

(The electrophysiological determinations were performed using standard protocols in the whole cell configuration of the patch-clamp technique (Hamill, O.P., Marty, A., Neher, E., Sakmann, B., Sigworth, F.J. Pflügers Arch- Eur. J. Physiol 391, 85 (1981)), with an extracellular solution containing (mM) 140 NaCl, 2.5 KCl, 2 CaCl2, 2 MgCl2, 10 Hepes/NaOH pH 7.2, 10 glucose. The pipette solution was (mM) 140 KCl, 10 BAPTA, 10 Hepes/ KOH pH 7.2.). The cells were treated overnight with antisense ODN 1 μM containing fluorescein-labeled ODN. The currents were determined 1 to 3 days later in cells showing fluorescence in their nuclei. The bars in Fig. 16C represent mean±S.E.M. for 9 cells (control) or 25 cells (antisense). Only the outward currents were evaluated in the analysis. Furthermore, the inhibition of DNA synthesis in human cancer cells (EFM-19, HeLa and SHSY-5Y) by antisense ODNs directed against h*eag* was investigated. DNA synthesis is expressed relative to BrdU incorporation in the absence of ODNs. The uptake conditions into cells using fluorescein labeled antisense ODN was optimized. Cells were seeded in 96-well plates at a density of 105 cells/ml. One day after plating, the cells were washed with culture medium and the ODN was added (final concentration 10 μM). The ODN had previously been mixed with 20 μg/ml of the transfection ragenent DAC-30 (Eurogentec) in serum-free medium and allowed to incubate at room temperature for 20-30 min. The mixture was then added as a 1:1 dilution in culture medium and maintained in contact with cells overnight. After this incubation, the cells were washed and labeled with BrdU (100μM) for 2 h. Incorporation was detected using the kit from Boehringer Mannheim and measured as OD units at 405 nm (reference 490 nm) after subtraction of the non-specific background incorporation. (Fig. 16D). The bars indicate mean±S.D. for eight wells per condition in a representative experiment.

Glossary and List of Abbreviations

Cell lines:

CHO	CHO-K1 (ATCC CCL 61)	Chinese hamster *Cricetulus griseus* ovary
HEK293	293 (ATCC CRL 1573)	Transformed primary human embryonal kidney
NIH3T3	(ATCC CRL 1658)	Embryo Swiss mouse fibroblasts
MCF7	(ATCC HTB 22)	Human breast adenocarcinoma
WT	Wild-type cells	

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Genes and gene products

eag	*ether-à-go-go* potassium channel
HERG	Human-*Eag*-Related Gene. Codes for an inwardly rectifying potassium channel mainly expressed in heart.
Kv1.4	Inactivating voltage-dependent potassium channel. Initially cloned from rat brain, it is present in many other tissues.

Others

EGF	Epidermal growth factor

PDGF	Platelet-derived growth factor
FCS	Fetal calf serum
I-V relation	Current-Voltage relation
LQT	Long Q-T (interval between Q and T waves in the electrocardiogram). Induces severe arrythmias due to repolarization defects.
BrdU	5-Bromo-2'-deoxyuridine. Structure analog of thymidine.
IC50	Concentration that produces 50% inhibition
RT-PCR. in	Polymerase Chain Reaction of cDNA produced by reverse transcription the same tube.

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